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FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **11752-002001**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **09/509306**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NZ98/00145	**25 September 1998**	**26 September 1997**

TITLE OF INVENTION: **THERAPEUTIC METHOD**

APPLICANT(S) FOR DO/EO/US: **REID, Ian Reginald; CORNISH, Jillian**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

"Express Mail" label number: EL445347431US
Date of Deposit : 23 March 2000
I hereby certify that under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Jonathan K Howard

PCTUS1/REV03

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/509306	PCT/NZ98/00145	11752-002001

20. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Search Report has been prepared by the EPO or JPO **$840.00**	
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) **$670.00**	
☐	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$760.00**	
☒	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO **$970.00**	
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) **$96.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$970.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	40 - 20 =	20	x $18.00	**$360.00**	
Independent claims	16 - 3 =	13	x $78.00	**$1,014.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	

TOTAL OF ABOVE CALCULATIONS =	**$2,344.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☐	**$0.00**	
SUBTOTAL =	**$2,344.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	**$0.00**	
TOTAL NATIONAL FEE =	**$2,344.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	**$0.00**	
TOTAL FEES ENCLOSED =	**$2,344.00**	
	Amount to be: refunded	$
	charged	$

☒ A check in the amount of **$2,344.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **06-1050** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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NAME

34,053
REGISTRATION NUMBER

23 March 2000
DATE

Attorney's Docket No.: 11752-002001 / 25703 MRB/smb

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Ian Reginald Reid et al.
Serial No. :
Filed : March 23, 2000
Title : THERAPEUTIC METHOD

Art Unit : Unknown
Examiner : Unknown

Box PCT

Assistant Commissioner for Patents
Washington, D.C. 20231

International Patent Application: PCT/NZ98/00145

International Filing Date: 25 September 1998

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Claims:

In claim 4, line 1, replace "any one of claims 1 to 3" with -- claim 1 --.

In claim 5, line 1, replace "any one of claims 1 to 3" with -- claim 1 --.

In claim 9, line 1, replace "any one of claims 6 to 8" with -- claim 6 --.

In claim 10, line 1, replace "any one of claims 6 to 8" with -- claim 6 --.

In claim 15, line 1, replace "any one of claims 12 to 14" with -- claim 12 --.

In claim 16, line 1, replace "any one of claims 13 to 15" with -- claim 13 --.

In claim 20, line 1, replace "any one of claims 17 to 19" with -- claim 17 --.

In claim 21, line 1, replace "any one of claims 17 to 19" with -- claim 17 --.

In claim 22, line 1, replace "any one of claims 17 to 19" with -- claim 17 --.

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL445347431US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

March 23, 2000
Date of Deposit

Signature

Jonathan R. Howard
Typed or Printed Name of Person Signing Certificate

In claim 26, line 1, replace "any one of claims 23 to 25" with -- claim 23 --.

In claim 27, line 1, replace "any one of claims 23 to 26" with -- claim 23 --.

In claim 28, line 1, replace "any one of claims 23 to 26" with -- claim 23 --.

In claim 29, line 1, replace "any one of claims 23 to 26" with -- claim 23 --.

In claim 31, line 1, replace "any one of claims 23 to 26" with -- claim 23 --.

In claim 32, line 1, replace "any one of claims 23 to 26" with -- claim 23 --.

Cancel claims 41 through 51 without prejudice.

REMARKS

Applicants have amended the dependency of the claims to preclude a rejection under 35 U.S.C. § 112, fifth paragraph, which provides in part that "[a] multiple dependent claim shall not serve as a basis for any other multiple dependent claim." No new matter has been added by the above amendments.

Applicant submits that all of the claims are now in condition for examination, which action is requested. Filed herewith is a check in payment of the excess claims fees required by the above amendments and a Petition for Automatic Extension with the required fee. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 3-23-00

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THERAPEUTIC METHOD

This invention is directed to new therapeutic uses which involve the stimulation of chondrocyte proliferation. More particularly, it is directed to the use of amylin and adrenomedullin as agents which stimulate chondrocyte proliferation and which therefore have utility in the treatment of cartilage disorders and/or cartilage mediated bone growth.

BACKGROUND

Amylin is a 37-amino acid peptide cosecreted with insulin from the beta cells of the pancreatic islets. It was first reported by Cooper *et al* in Proceedings of the National Academy of Sciences, USA 84, 8628 (1987) and is the subject of European Patent 289287. Amylin has the following peptide sequence:

Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-
1 5 10

Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-
11 15 20

Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-
21 25 20

Asn-Val-Gly-Ser-Asn-Thr-Tyr
31 35

The native molecule contains a disulphide bridge between the cysteine residues shown at positions 2 and 7 in the primary structure, is amidated at its COOH-terminus, and is formed as a propeptide.

European Patent 289287 reports a number of biological effects including enhancement of hepatic glucose output, increased production of lactate from skeletal muscle and reduced action of insulin in skeletal muscle.

Amylin is also reported in European Patent 408284 as having value for treatment of bone disorders and calcium imbalance. The patent specification attributes the activity of amylin to an inhibition of osteoclast motility. It is also reported in WO 96/02269 as stimulating bone growth through stimulating osteoblast proliferation.

Adrenomedullin is a 52-amino acid peptide first described in 1993 by Kitamura *et al* (Kitamura, K., *et al.* Adrenomedullin, a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem. Biophys. Res. Commun.* 192:553-560 (1993)). It is present in normal adrenal/medulla and in many other tissues including the atria, ventricles, endothelial cells, lungs, brain, kidneys and bone.

Adrenomedullin shows approximately 20% sequence identity with amylin and can therefore be termed a related peptide (Muff, R., *et al.* Calcitonin, calcitonin gene-related peptide, adrenomedullin and amylin: homologous peptides, separate receptors and overlapping biological actions. *Eur. J. Endocrinol.* 133:17-20 (1995)). Both peptides have an NH_2 terminal ring created by a disulphide bond and are amidated at their COOH terminals.

Like amylin, adrenomedullin is also reported to have a range of activities. It is a potent vasodilator. It also has value in the treatment of bone disorders. This is primarily through an ability to stimulate osetoblast activity and proliferation *in vitro* and *in vivo* (Cornish, J., *et al.* Adrenomedullin is a potent stimulator of osteoblastic activity *in vitro* and *in vivo*. *Am. J. Physiol (Endocrinol Metab)* 36:E1113-E1120, (1997)).

However, to date, there has been no report of either of the peptides amylin or adrenomedullin, as having any effect on chondrocytes. It is the applicants finding that both of these peptides are effective in the stimulation of chondrocyte proliferation and therefore on the growth of both cartilage and lineal bone. This effect is believed to be mediated through a single receptor on chrondrocytes which underlies the applicant's invention.

SUMMARY OF THE INVENTION

The invention has a number of aspects. In a first aspect, the invention provides a method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of increasing the active concentration of amylin within said patient.

Another aspect provides a method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of administering to said patient amylin or an analog thereof in an amount effective to stimulate chondrocyte proliferation.

In another embodiment, the invention provides a method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of increasing the active concentration of adrenomedullin within said patient.

In a further embodiment, the invention provides a method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of administering to said patient adrenomedullin or an analog thereof in an amount effective to stimule chondrocyte proliferation.

In still a further aspect, the invention provides a method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of activating the receptor localised on chondrocytes of said patient to which amylin and/or adrenomedullin bind.

Most preferably, the method involves activation of the adrenomedullin receptor.

Conveniently, in each of the above methods the stimulation of chondrocyte proliferation is part of a method of treating a patient to stimulate cartilage growth and/or repair or to stimulate bone growth.

The invention also provides a method of stimulating chondrocyte proliferation *in vitro* which comprises administering an amount of amylin, adrenomedullin or an analog of either amylin or adrenomedullin to said chondrocytes which is effective in inducing chondrocyte proliferation.

Other aspects include

the use of amylin or an analog thereof in the preparation of a medicament for effecting chondrocyte proliferation;

the use of adrenomedullin or an analog thereof in the preparation of a medicament for effecting chondrocyte proliferation;

the use of a ligand which binds to and activates the receptor to which amylin and/or adrenomedullin binds (preferably the adrenomedullin receptor) in the preparation of a medicament for effecting chondrocyte proliferation;

the use of an amylin agonist in the preparation of a medicament for effecting chondrocyte proliferation;

the use of an adrenomedullin agonist in the preparation of a medicament for effecting chondrocyte proliferation;

the use of amylin-(1-8) in the preparation of a medicament for effecting chondrocyte proliferation; and

the use of adrenomedullin-(27-52) in the preparation of a medicament for effecting chondrocyte proliferation.

DESCRIPTION OF THE DRAWINGS

The present invention is broadly as defined above. However, it will be appreciated by those persons skilled in the art that it is not limited thereto and that it also includes embodiments which are more particularly described below and illustrated by the experimental data presented. This data includes the information shown in the accompanying drawings in which:

Figure 1 shows the effects of daily systemic administration of amylin for 4 weeks on growth plate width in the tibiae of normal adult male mice. n = 20 in each group. *, significantly different from control, P = 0.0002;

Figure 2 shows the effects of daily systemic administration of amylin for 4 weeks on bone length in the tibiae of normal adult male mice. n = 20 in each group. *, significantly different from control, P = 0.004;

Figure 3 shows the effect of the amylin fragment (amylin (1-8)) on epiphyseal growth plate width; and

Figure 4 shows the effect of the adrenomedullin fragment (adm 27-52) on epiphyseal growth plate width.

DESCRIPTION OF THE INVENTION

As broadly defined above, the present invention relates primarily to methods for stimulating chondrocyte proliferation. The invention therefore has utility in any application where stimulation of chondrocyte proliferation or growth is viewed as desirable, including for example cartilage growth and bone growth.

The applicants have found that chondrocyte proliferation is able to be effected using a number of related approaches. A first such approach is through a focus upon amylin. The applicants have found that increasing the effective concentration of amylin within a patient able to interact with the patients chondrocytes has the effect of stimulating chondrocyte proliferation.

Amylin for use in accordance with this approach can be obtained from any convenient commercial source (such as Bachem California, Torrence, CA, USA). Alternatively, amylin can be synthesised, using the procedure as described by way of example in EP 408284.

The amylin used can be homologous or heterologous to the patient to be treated. For example, amylin from humans and other mammals eg. rat, monkey, dog, cat, mouse, guinea pig, hamster, degus, rabbit and hare can be used. The structure of

these various peptides is reported in *Endocrine Reviews* 1994, 15(2) 163 by Garth J S Cooper which is incorporated herein by reference.

Most conveniently, the effective concentration of amylin will be increased through direct administration using either amylin itself or an amylin pro-drug (a form which is cleaved within the body to release amylin). It is however not the applicants intention to exclude increasing amylin concentration through administration of either amylin agonists (substances which effect a direct increase in the production or activity of amylin within the body, or inhibitors of amylin antagonists (substances which bind amylin or otherwise prevent or reduce the action of amylin within the body. These latter compounds exert an indirect effect on effective amylin concentrations through the removal of an inhibitory mechanism.

Another possibility is administration of a replicable vehicle encoding amylin to the patient. Such a vehicle (which may be a modified cell line or virus which expresses amylin within the patient) could have application in increasing the concentration of amylin within the patient for a prolonged period.

It is also contemplated that amylin analogs can be employed in this invention. As used herein "analog" means a protein which is a variant of another protein through insertion, deletion or substitution of one or more amino acids but which retains at least substantial functional equivalency.

A protein is a functional equivalent of another protein for a specific function if the equivalent protein is immunologically cross-reactive with, and has at least substantially the same function as, the original protein. The equivalent can be, for example, a fragment of the protein, a fusion of the protein with another protein or carrier, or a fusion of a fragment with additional amino acids. For example, it is possible to substitute amino acid in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids normally held to be equivalent are:

(a) Ala, Ser, Thr, Pro, Gly;

(b) Asn, Asp, Glu, Gln;

(c) His, Arg, Lys;

(d) Met, Leu, Ile, Val; and

(e) Phe, Tyr, Trp.

In the case of amylin, the preferred analogs are fragments of the protein. In particular, amylin (1-8) can be used (ie. a fragment consisting of amino acids 1 to 8 of the amylin sequence).

Functional equivalency of analogs can also be readily screened for by reference to the ability of the analog to both bind to and activate the appropriate receptor.

In addition to the above approach, which focuses upon amylin and its analogs, the invention provides a further approach to chondrocyte proliferation. This second approach has a focus upon adrenomedullin. The applicants have found that, in an equivalent manner to amylin, increasing the effective concentration of adrenomedullin within a patient able to interact with the chondrocytes in that patient stimulates chondrocyte proliferation.

For use in this approach, adrenomedullin can be obtained from any convenient commercial source or, as is the case with amylin, synthesised using techniques well known in the art. Such techniques include those described hereinafter.

Again, it is most convenient that the effective concentration of adrenomedullin be increased through direct administration using either adrenomedullin itself or an adrenomedullin pro-drug. However, adrenomedullin agonists or inhibitors of adrenomedullin antagonists are not excluded.

As with amylin, adrenomedullin can also be administered in the form of a replicable vehicle encoding adrenomedullin to the patient for release of adrenomedullin over a prolonged period.

Adrenomedullin analogs can also be employed. For this purpose, the term "analog" has the equivalent meaning of that given above for amylin. In the case of adrenomedullin, a particularly preferred analog is adrenomedullin (27-52) (ie. a fragment consisting of amino acids 27-52 of the adrenomedullin sequence).

The invention still further provides a third approach to chondrocyte proliferation. This further approach focuses upon the receptors on chondrocytes to which amylin

and adrenomedullin bind and upon effecting chondrocyte proliferation through use of any ligand which both binds to and activates these receptors.

It will be appreciated that amylin, analogs of amylin, adrenomedullin and analogs of adrenomedullin are all ligands which achieve this Indeed, the use of these substances as active agents represents a preferred aspect of the invention. However, it should be appreciated that this approach has not restricted the use of amylin, adrenomedullin and their analogs but also extends to any ligand which fulfils the functional requirement of both binding to and activating (stimulating) the amylin or adrenomedullin receptors. Such additional ligands are, for example, believed to include peptides such as calcitonin gene related peptide (Muff, R., *et al.* Calcitonin, calcitonin gene-related peptide, adrenomedullin and amylin: homologous peptides, separate receptors and overlapping biological actions. *Eur. J. Endocrinol.* 133:17-20 (1995)).

A specific feature of this approach is to employ ligands which bind to and activate the adrenomedullin receptor. This receptor was described in, for example, Kapas, S., *et al.* Cloning and expression of cDNA encoding a rat adrenomedullin receptor. *J. Biol. Chem.* 270:25344-25347 (1995). It is further described in Montuenga, L. M., *et al.* Expression of adrenomedullin and its receptor during embryogenesis suggests autocrine or paracrine modes of action. *Endocrinology* 138:440-451 (1997)).

Additional stimulatory ligands can therefore, for example, be identified by a screening protocol employing at least the ligand binding domain of the adrenomedullin receptor. This screening method can, for example, utilise the expression of the adrenomedullin receptor in Xenopus oocytes using standard recombinant DNA methods and measurement of the adrenomedullin receptor-mediated signal transduction evoked by novel stimulatory ligands.

For therapeutic application, the active compound (amylin, adrenomedullin, analog or ligand) will be formulated as a medicament. The details of the formulation will ultimately depend upon the active compound itself and upon the route of administration chosen. It will however be usual for the medicament to include combination of the active compound with a suitable carrier, vehicle or diluent.

Dosage rates will also be active compound and administration route dependent. However, by way of example, the dosage of active compound to be administered by injection will be in the range of 0.01-100 mg/kg of body weight.

Further, while formulations in which the active compounds represent the sole active principle are most likely to be used, it is by no means intended that formulations which are suitable for combination therapies be excluded. The active compound can be administered together with any other therapeutic agent, including any other agent which has an effect on chondrocyte proliferation.

The invention, in its various aspects, will now be illustrated by the experimental section which follows. It will however be appreciated that the experiments are non-limiting.

EXPERIMENTAL

METHODS

(a) Chondrocyte Monolayer Cell Cultures

Fresh cartilage samples were collected from the tibial plateaus and femoral condyles of mature, healthy crossbred dogs (2-4 years; 20-25 kg). The chondrocytes were isolated as previously described (Connective Tissue Research 1988; 18:205-222). Briefly, the chondrocytes were obtained by pronase and collagenase digestion of the cartilage, then the cells were centrifuged, washed and resuspended in media before being cultured in 75 cm^2 tissue culture flasks. The cells were incubated under 5% CO_2 and 95% air at 37°C. Confluence was reached by 7-10 days, at which time the cells were subcultured. After trypsinization, the cells are rinsed and resuspended in fresh medium, then seeded at 5 x 10^4 cells/ml in 24-well plates (0.5 ml cell suspension per well, ie. 1.4 x 10^4 cells/cm^2). *Proliferation studies* (cell counts and thymidine incorporation) were performed. Subconfluent population were changed to serum-free medium with 0.1% bovine serum albumin plus the experimental compounds. Cell numbers were analysed at 24 hours after addition of the peptide or vehicle. The cell numbers were determined using a haemocytometer chamber. Results were expressed per well. [^{3}H]-*thymidine incorporation* was assessed by pulsing the cells with [^{3}H]-thymidine (1uCi/well) two hours before the end of the experimental incubation. The experiment was terminated at 24 hours by washing

the cells in media containing cold thymidine followed by the addition of 10% tricholoroacetic acid. The precipitate was washed twice with ethanol:ether (3:1) and the wells desiccated at room temperature. The residue was redissolved in 2 M KOH at 55°C for 30 mins, neutralised with 1 M HCl, and an aliquot counted for radioactivity. Results were expressed as dpm per well. For both cell counts and thymidine incorporation, each experiment was performed at least 4 different times using experimental groups consisting of at least 6 wells.

(b) Chondrocytes 3-dimensional cell cultures in alginate beads

Alginate head cultures were established as described by Guo, *et al.* Culture and growth characteristics of chondrocytes encapsulated in alginated beads. *Connective Tissue Research.* 19:277-297 (1989). Briefly the cells were suspended in a solution of 1.25% (wt/vol) alginate in HEPES (20 mM HEPES buffer pH neutral) at a density of $2x10^6$ cells/ml> The suspension of chondrocytes were slowly extruded through a 22-gauge needle in a dropwise manner into 40 ml of 0.1 M $CaCl_2$ solution. After instantaneous gelation, the beads were allowed to further polymerise in $CaCL_2$ solution (10 mins, room temperature). The beads were washed sequentially, twice in 0.15 M NaCl and twice in Dulbecco's modified Eagle's medium (DME). After the washing procedure, the beads were placed into 24-well culture plates (10 beads/well) and fed with 1ml 10% fetal calf serum (FCS) SMW with 5µg/ml ascorbic acid. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every second day. On day 4 and 6 of culture, peptide or vehicle was added. Cell numbers were analysed at day 8 by exposing alginate beads to 50 mM ethylenediaminetetraacetic acid (EDTA) for approximately 10 minutes at 37°C. Counting was performed in a haemocytometer chamber. Results were expressed per well. Tritiated-thymidine incorporation (^{3}H-thymidine) was assessed by pulsing the beads with ^{3}H-thymidine (1µCi/well) 48 hours before the end of experimental incubation. Experiments were then terminated at day 8 of culture by dissolving the beads in 50 mM EDTA. The cells were washed twice with distilled water by centrifuging. Pellets were resuspended and counted for radioactivity.

(c) *In Vivo* Study: Experimental Design

Two groups of 20 sexually mature male Swiss mice aged between 40 and 50 days and weighing 25-32g, were given daily subcutaneous injections (50 ul) in the loose skin at the nape of the neck for 5 days/week over 4 consecutive weeks. The treated

group was injected with peptide at a dose of 300 ug/kg/injection and the control group was injected with vehicle (water). Animals were housed in a room maintained at 20°C on 12-hour light/dark cycles. They were fed diet 86 rodent pellets (New Zealand Stockfeed Ltd) ad libitum throughout the experiment. Each animal's weight was recorded at the beginning and end of the experiment. One day after the last injection, animals were sacrificed by cervical dislocation. They study had the approval of the local institutional review board.

The tibiae were dissected free of adherent tissue. Tibial lengths were recorded by measuring the distance between the proximal epiphysis and the distal tibio-fibular junction using an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Bones were placed in 10% phosphate-buffered formalin for 24 hours and then dehydrated in a graded series of ethanol solutions and embedded, undecalcified, in methylmethacrylate resin. Tibiae were sectioned longitudinally through the frontal plane and calvariae were cut cross-sectionally at the base of the parietal bone. All sections were 4 um think and were cut on a Leitz microtome using a tungsten-carbide knife (Microknife Sharpening, Utah, USA). Sections were mounted on gelatin-coated slides and air-dried. They were stained with Goldner's tri-chrome and examined using an Olympus BX 50 microscope (Olympus Optical Co Ltd, Tokyo, Japan) which was attached to an Osteomeasure Image Analyzer (Osteometrics Inc. Atlanta, GA).

Tibial histomorphometric analyses were made from three adjacent sections one third of the way through the anterior/posterior depth of the proximal tibiae. Epiphyseal growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator who was blinded to the treatment group of each bone.

Materials

Rat amylin was sourced from Bachem California, Torrance, CA, USA. Lyophilised material was dissolved in water prior to administration. Methylmethacrylate was purchased from Acros Organics N.V., Geel, Belgium.

Rat amylin-(1-8) used in this study was a COOH-terminal amide synthesized on methylbenzhydrylamine resin by standard solid-phase techniques followed by hydrogen fluoride deprotection and cleavage from the resin. Amylin-(1-8) was

cyclized in a dilute solution of 90% acetic acid by treatment with methanol solutions of iodine and purified to >96% homogeneity by reverse-phase high performance liquid chromatograph (RP HPLC). Structures were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF system, model G2025 A, Hewlett Packard CA, USA) and amino acid analysis of acid hydrolysates %%4929%%.

Human adrenomedullin and its fragments were synthesized on methylbenzhydrylamine resin using standard solid-phase procedures, and cleaved with hydrogen fluoride/anisole. Sequences containing a disulfide bridge were cyclized by titration with I_2 in 90% acetic acid/water solutions. Crude materials were purified by gel filtration on Sephadex columns in 50% acetic acid followed by gradient elution on C18 silica using acetonitrile/0.1% trifluoroacetic acid eluants. Homogeneity of final peptides was assessed by thin layer chromatography, analytical HPLC, amino acid analysis and matrix-assisted laser-desorption-ionization mass spectroscopy. Purities were usually >98%.

Statistical Analysis

Data are presented as mean ± sem. Where parameters have been measured more than once in each animal these values have been averaged to produce a single value for each animal before further analysis. The significant of treatment effects was evaluated using Student's *t* tests for unpaired data. These comparisons were specified a priori, so adjustment of $\alpha(0.05)$ was not necessary.

RESULTS

Amylin

(a) Chondrocyte Cell Cultures

Amylin influenced chondrocyte proliferation, increasing cell numbers from 4.12 ± 0.23 ($x10^4$) (mean ± sem) in control cells to 5.11 ± 0.21 ($x10^4$) in those cells incubated with amylin ($p=0.01$) as well as increasing thymidine incorporation (ie. DNA synthesis) from 20725 ± 997 dpm in control cells to 25937 ± 1203 dpm in amylin-treated cells.

(b) Chondrocytes 3-dimensional cell cultures in alginate beads

Amylin again influenced chondrocyte proliferation, increasing cell numbers from 5.58 ± 0.16 ($x10^4$) (mean ± sem) in control cells to 6.07 ± 0.05 ($x10^4$) in those cells incubated with amylin (10^{-10}M) ($p<0.03$) as well as increasing thymidine incorporation (ie. DNA synthesis) from 1135 ± 85 dpm in control cells to 2584 ±229 dpm in amylin-treated cells ($p<0.001$).

(c) *In Vivo* Study

Amylin influenced the tibial growth plate, increasing its width from 0.083 ± 0.005 mm (mean ± sem) in the control animals to 0.108 ± 0.003 mm in those receiving amylin (P = 0.0002) (Figure 1). The total length of the tibiae was also increased from 11.31 ± 0.07 mm in control animals to 11.67 ± 0.09 mm in animals injected with amylin (P-0.004) (Figure 2).

Amylin 1-8

(a) Amylin-(1-8) also influenced chondrocyte proliferation, increasing cell numbers from 3.23 ± 0.11 ($x10^4$) (mean ± sem) in control cells to 3.63 ± 0.09 ($x10^4$) in those cells incubated with amylin-(1-8) (10-8M) (p = 0.02) as well as increasing thymidine incorporation (DNA synthesis) from 26859 ± 423 dpm in control cells to 28932 ±628 dpm in amylin=(1-8) treated cells (p = 0.02).

(c) The growth plate width in the proximal tibiae of mice injected systemically with amylin-(1-8) is significantly increased compared to control animals (mean ± sem: 0.111 mm ± 0.004 compared to 0.081 mm ± 0.004; $p<0.0001$). See Figure 3.

Adrenomedullin

(a) Adrenomedullin influenced chondrocyte proliferation, increasing cell numbers from 1.79 ± 0.07 (x 10^4) (mean ± sem) in control cells to 2.27 ± 0.12 (x 10^4) in those cells incubated with adenomedullin (10^{-9}M) ($p<0.01$).

Adrenomedullin-(27-52)

(c) Adrenomedullin-(27-52) increased the growth plate width from 0.094 mm ± 0.003 (mean ± sem) in control animals to 0.11 mm ± 0.003 in adrenomedullin-(27-52) ($p=0.003$). See Figure 4.

INDUSTRIAL APPLICATION

The above results clearly show that amylin and its anlogs (amylin-(1-8), for example) has the ability to stimulate chondrocyte proliferation. Similarly, adrenomedullin and its analogs (adrenomedullin-(27-52) have equivalent capability.

The results additionally show the ability of both amylin, adrenomedullin and their analogs to influence the growth of cartilage as well as increased bone growth. This latter effect is consistent with the formation of bone on a template of cartilage tissue.

Both amylin and adrenomedullin are believed to be exerting the effect on chondrocyte proliferation/cartilage growth/bone formation through the mediation of the amylin/adrenomedullin receptor.

The present invention therefore provides new approaches to chondrocyte proliferation. These involve firstly increasing the active concentration of amylin/adrenomedullin in a patient and secondly the activation of the amylin/adrenomedullin receptor localised on chondrocyte cells.

The approaches of the invention have application in the treatment of patients in a variety of conditions. Principal amongst these are conditions where the patient is suffering from a cartilage defect, either through injury or through degenerative, inflammatory or other disease.

The approaches of the invention also have application in the stimulation of bone growth, particularly lineal bone growth. This provides the invention with application in treating patients (for example, children) who are of short stature or who otherwise suffer from defects which would benefit from stimulation of the growth of limb bones.

The invention also has application *in vitro*. Extracted chondrocytes can be proliferated using the present methods. The proliferated chondrocytes can then be employed in methods of therapy, particularly those which involve the treatment of damaged cartilage.

It will be appreciated by those persons skilled in the art that the above description is provided by way of example only and that numerous changes and variations can be made while still being within the scope of the invention as defined by the appended claims.

CLAIMS

1. A method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of increasing the active concentration of amylin within said patient.

2. A method of treating a patient to stimulate cartilage growth and/or repair *in vivo* through stimulation of chondrocyte proliferation which comprises the step of increasing the active concentration of amylin within said patient.

3. A method of treating a patient to stimulate bone growth *in vivo* through stimulation of chondrocyte proliferation which comprises the step of increasing the active concentration of amylin within said patient.

4. A method according to any one of claims 1 to 3 wherein the active concentration of amylin is increased through administration of amylin to said patient.

5. A method according to any one of claims 1 to 3 wherein the active concentration of amylin is increased through administration of an amylin agonist.

6. A method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of administering to said patient amylin or an analog thereof in an amount effective to stimulate chondrocyte proliferation.

7. A method of treating a patient to stimulate cartilage growth and/or repair *in vivo* through stimulation of chondrocyte proliferation which comprises the step of administering to said patient amylin or an analog thereof in an amount effective to stimulate chondrocyte proliferation.

8. A method of treating a patient to stimulate bone growth *in vivo* through stimulation of chondrocyte proliferation which comprises the step of administering to said patient amylin or an analog thereof in an amount effective to stimulate chondrocyte proliferation.

9. A method according to any one of claims 6 to 8 wherein amylin is administered to said patient.

10. A method according to any one of claims 6 to 8 wherein an analog of amylin is administered to said patient

11. A method according to claim 10 wherein said amylin analog is amylin-(1-8)

12. A method of treating a patient to stimulate chondrocyte proliferation *in vitro* which comprises the step of increasing the active concentration of adrenomedullin within said patient.

13. A method of treating a patient to stimulate cartilage growth and/or repair *in vivo* through stimulation of chondrocyte proliferation which comprises the step of increasing the active concentration of adrenomedullin within said patient.

14. A method of treating a patient to stimulate both growth *in vivo* through stimulation of chondrocyte proliferation which comprises the step of increasing the active concentration of adrenomedullin within said patient.

15. A method according to any one of claims 12 to 14 wherein the active concentration of adrenomedullin is increased through administration of adrenomedullin to said patient.

16. A method according to any one of claims 13 to 15 wherein the active concentration of adrenomedullin is increased through administration of an adrenomedullin agonist.

17. A method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of administering to said patient adrenomedullin or an analog thereof in an amount effective to stimule chondrocyte proliferation.

18. A method of treating a patient to stimulate cartilage growth and/or repair *in vivo* through stimulation of chondrocyte proliferation which comprises the step of administering to said patient adrenomedullin or an analog thereof in an amount effective to stimulate chondrocyte proliferation.

19. A method of treating a patient to stimulate bone growth *in vivo* through stimulation of chondrocyte proliferation which comprises the step of

administering to said patient adrenomedullin or an analog thereof in an amount effective to stimulate chondrocyte proliferation

20. A method according to any one of claims 17 to 19 wherein adrenomedullin is administered to said patient.

21. A method according to any one of claims 17 to 19 wherein an analog of adrenomedullin is administered to said patient

22. A method according to any one of claims 17 to 19 wherein said adrenomedullin analog is adrenomedullin-(27-52).

23. A method of treating a patient to stimulate chondrocyte proliferation *in vivo* which comprises the step of activating a receptor localised on chondrocytes of said patient to which amylin and/or adrenomedullin binds.

24. A method of treating a patient to stimulate cartilage growth and/or repair *in vivo* through stimulation of chondrocyte proliferation which comprises the step of activating a receptor localised on chondrocytes of said patient to which amylin and/or adrenomedullin binds.

25. A method of treating a patient to stimulate bone growth *in vivo* through stimulation of chondrocyte proliferation which comprises the step of activating a receptor localised on chondrocytes of said patient to which amylin and/or adrenomedullin binds.

26. A method according to any one of claims 23 to 25 wherein the receptor which is activated is the adrenomedullin (ADM) receptor.

27. A method according to any one of claims 23 to 26 wherein receptor activation is effected through administration of a ligand which binds to and activates the receptor.

28. A method according to any one of claims 23 to 26 wherein receptor activation is effected through administration of amylin.

29. A method according to any one of claims 23 to 26 wherein receptor activation is effected through administration of an amylin analog.

30. A method according to claim 29 wherein the amylin analog is amylin-(1-8).

31. A method according to any one of claims 23 to 26 wherein ADM receptor activation is effected through administration of adrenomedullin.

32. A method according to any one of claims 23 to 26 wherein receptor activation is effected through administration of an adrenomedullin analog

33. A method according to claim 32 wherein the adrenomedullin analog is adrenomedullin-(27-52).

34. A method of stimulating chondrocyte proliferation *in vitro* which comprises administering an amount of amylin, adrenomedullin or an analog of either amylin or adrenomedullin to said chondrocytes which is effective in inducing chondrocyte proliferation.

35. A method according to claim 34 wherein an effective amount of amylin is administered.

36. A method according to claim 34 wherein an effective amount of an amylin analog is administered.

37. A method according to claim 36 wherein the amylin analog is amylin-1-8.

38. A method according to claim 34 wherein an effective amount of adrenomedullin is administered.

39. A method according to claim 34 wherein an effective amount of an adrenomedullin analog is administered.

40. A method according to claim 39 wherein the adrenomedullin analog is adrenomedullin-27-52.

41. The use of amylin or an analog thereof in the preparation of a medicament for effecting chondrocyte proliferation.

42. The use of adrenomedullin or an analog thereof in the preparation of a medicament for effecting chondrocyte proliferation.

43. The use of a ligand which binds to and activates a receptor localised on chondrocytes to which amylin and/or adrenomedullin binds in the preparation of a medicament for effecting chondrocyte proliferation.

44. The use of claim 43 wherein the ligand is one which binds to and activates the adrenomedullin (ADM) receptor.

45. The use of any one of claims 41 to 44 wherein the medicament is for the growth and/or repair of cartilage.

46 The use of any one of claims 41 to 44 wherein the medicament is for the growth of bone.

47. The use of claim 46 wherein the medicament is for effecting the lineal growth of bone.

48. The use of an amylin agonist in the preparation of a medicament for effecting chondrocyte proliferation.

49. The use of an adrenomedullin agonist in the preparation of a medicament for effecting chondrocyte proliferation.

50. The use of amylin-(1-8) in the preparation of a medicament for effecting chondrocyte proliferation.

51. The use of adrenomedullin-(27-52) in the preparation of a medicament for effecting chondrocyte proliferation.

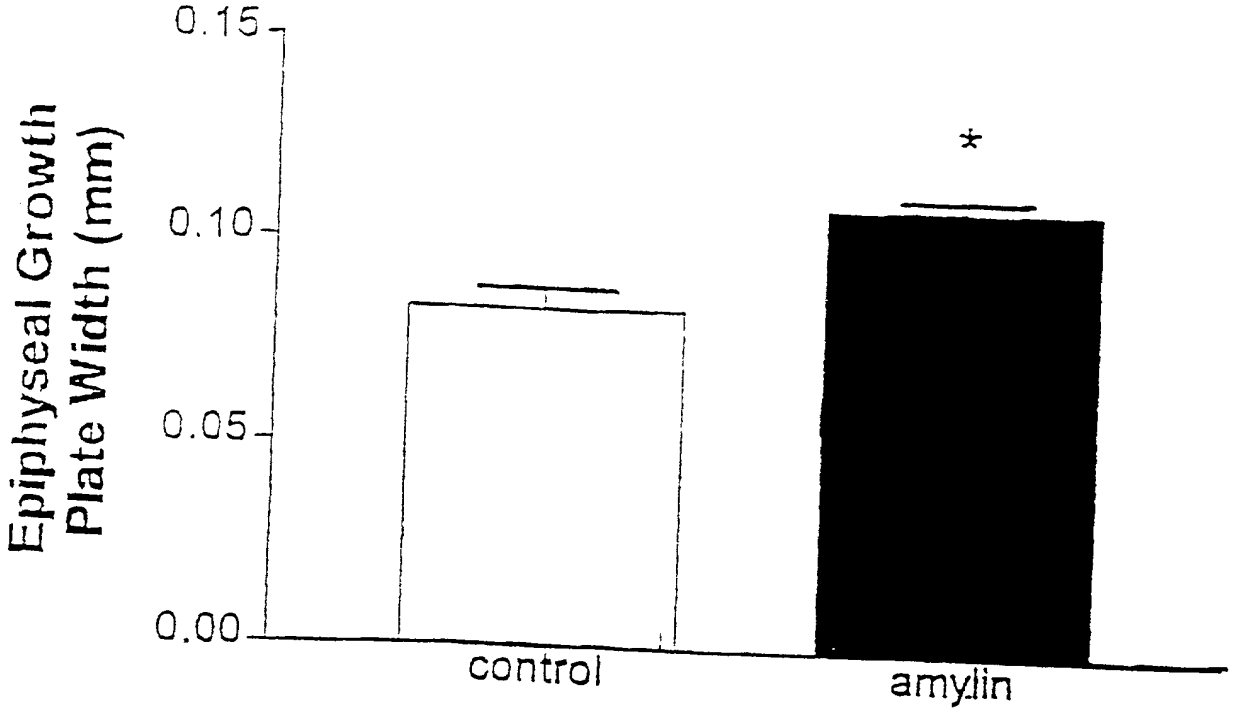

FIGURE 1

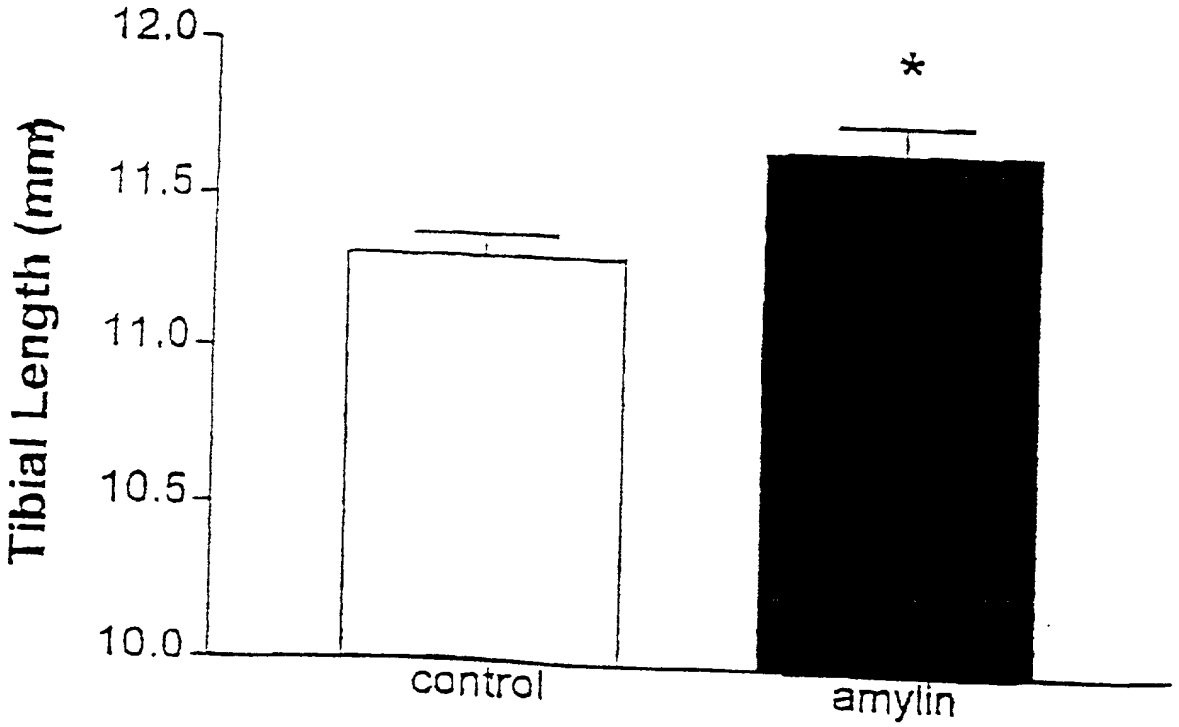

FIGURE 2

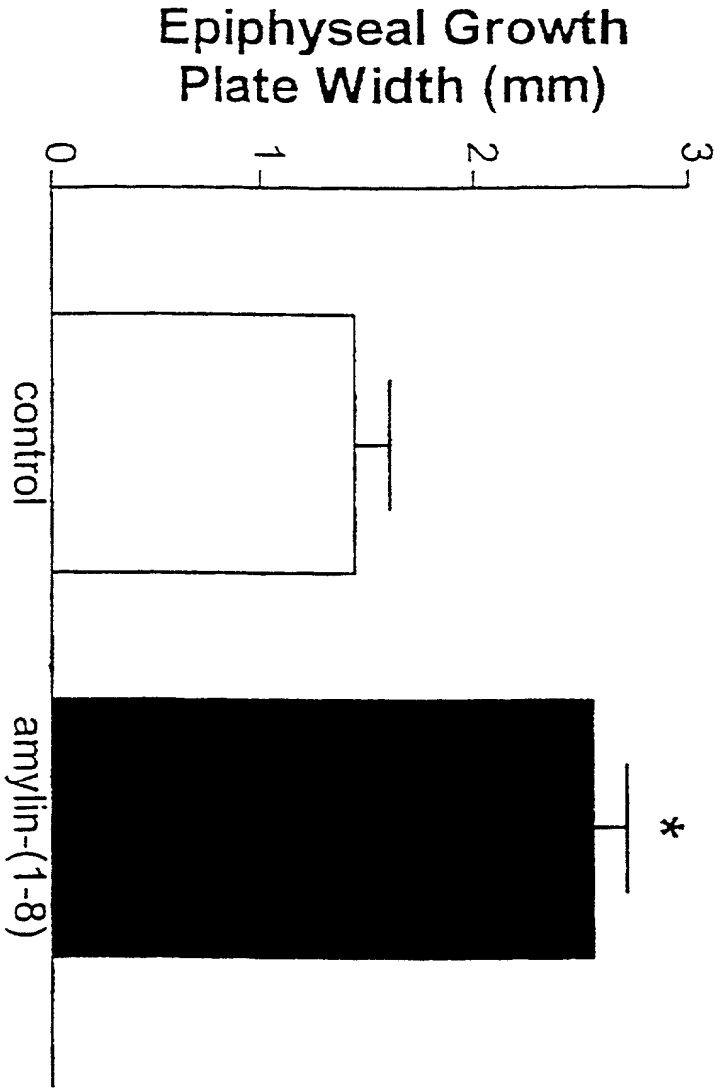

FIGURE 3

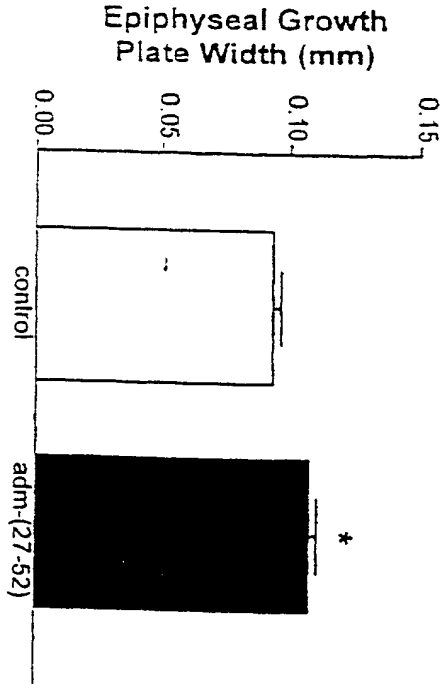
Epiphyseal Growth
Plate Width (mm)
0.00
0.05
0.10
0.15
control
adm-(27-52)
*

FIGURE 4

Attorney's Docket No.: Auckland-002001 YRT/JRG
Client's Ref.: 25703 MRB/smb

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled THERAPEUTIC METHOD, the specification of which:

- [X] is attached hereto.
- [ ] was filed on _ as Application Serial No. _ and was amended on _____.
- [ ] was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
PCT	PCT/NZ98/00145	September 25, 1998	[X] Yes [ ] No
New Zealand	328853	September 26, 1997	[X] Yes [ ] No

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Combined Declaration and Power of Attorney
Page 2 of 2 Pages

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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